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Research Article

Free radical scavenging activity of extracts from seaweeds *Macrocystis pyrifera* and *Undaria pinnatifida*: applications as functional food in the diet of prawn *Artemesia longinaris*

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ABSTRACT. Water-soluble extracts from seaweeds *Macrocystis pyrifera* (M) and *Undaria pinnatifida* (U) were prepared to use the polysaccharide bioactive fraction as functional food. Chemical analysis showed that the main components of both extracts were mannitol and fucoidans. Also in both cases minor amounts of rhamnose, glucose, xylose and arabinose were determine. Content of fucose+galactose was higher in *U. pinnatifida* (8.7%) than in *M. pyrifera* (5.9%). Extracts were used as feed additive to *Artemesia longinaris* at inclusion levels of 1 and 2% (M1 and M2; U1 and U2), a diet without extract was used as control. Radical scavenging activities were estimated for two extracts and for midgut gland samples of experimental animals. Scavenging activity was detected in both extracts, the signal decayed to 50 and 62% in 18 min for *U. pinnatifida* and *M. pyrifera*, respectively. After 30 days of feeding, results did not show variation of survival, but growth was improved in animals fed with U1 and U2. Supplementation with 1 and 2% of *U. pinnatifida* showed similar quenching capacity of homogenates than those fed with M2 with a rapid 1,1-diphenylpicrylhydrazyl (DPPH) decay at 2 min of reaction. Recommended concentration for culture *A. longinaris* is 2%, as it is that produce the best antioxidant capacity, although the growth performance was improved also significantly only with *U. pinnatifida* extract.

Keywords: bioactive compounds, seaweeds, free radicals, nutrition, shrimp.

INTRODUCTION

Biological properties of algae have been summarized in numerous reviews. The most recent studies deal with nutritional (Jiménez-Escrig *et al*., 2001; Kumar *et al*., 2008; Jiao *et al*., 2011) and pharmacological (Cardozo *et al*., 2007; Lordan *et al*., 2011; Thomas & Kim, 2011) applications. Global marine algae utilization is a multibillion-dollar industry; however, it is mainly limited to farming of edible species or production of the hydrocolloids agar, carrageenan, and alginates. Marine algae are also rich in dietary fibers, sulfated polysaccharides, omega-3 fatty acids, amino acids, bioactive peptides, vitamins, minerals, and carotenoids (Kadam *et al*., 2013). The bioactive potential of marine algae as a functional food ingredient is underexploited; however, many initiatives are currently being taken to exploit them as food ingredient applications. Moreover, polysaccharides of algal origin such as carrageenan, laminaran, alginate, and fucoidans have also exhibited immunomodulatory effects on shrimp. Information regarding the effects of seaweed polysaccharides on shrimp physiology has been relatively scarce (Traifalgar *et al*., 2012).

The cell wall of brown algae is mainly composed of fucoidan, alginate and laminarin and their derivatives in variable proportions. These polysaccharides provide strength and flexibility, maintain ionic equilibrium and prevent for desiccation. Fucoidans are complex sulfated polysaccharides consisting mainly of sulfated L-fucose and variable proportions of galactose, mannose, xylose, glucose, rhamnose and uronic acids (Usov & Bilan, 2009). In vertebrates, these polysaccharides have been documented to elicit a wide range of biological activities, such as anticancer, anticoagulant, antiviral, and immune-enhancing properties. Although the biolo-

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gical effects of fucoidans on vertebrates are well established, their effects on commercially important aquatic animals, specifically on shrimp, are not fully evaluated (Traifalgar *et al*., 2010).

The use of brown algae extracts as functional shrimp feed, may be beneficial due to their antioxidant activity, which is similar to α-tocopherol (vitamin E). Its antioxidant property is attributed to the presence of polar compounds that prevent the uptake of oxygen by the substrate, inhibiting the formation of peroxides and/or acting as a donor of electrons or hydrogen atoms. Excessive production and/or exposure of an organism to free radicals induce cell damage, brought about by the excessive production of reactive oxygen species (ROS) and free radicals (Sánchez-Rodríguez *et al*., 2004).

Recently, algal polysaccharides have been demonstrated to play an important role as free radical scavengers *in vitro* for the prevention of oxidative damage in living organisms. *In vitro* antioxidant action is used as a first tool to select potential compounds for protection from lipid oxidation and for further formulation of functional feeds (Balboa *et al*., 2013). DPPH is a stable free radical widely used for evaluating natural antioxidants (algae or algal products) (Kang *et al*., 2003; Kuda *et al*., 2007) due to its stability, simplicity and reproducibility.

Seaweed meals have been used as food additives for several aquatic organisms to promote growth and feed efficiency. In the white shrimp (*Litopenaeus vannamei*) inclusion of meal of *Macrocystis pyrifera* (Cruz-Suárez *et al*., 2009) and *Sargassum* spp. (Casas-Valdez *et al*., 2002) at concentrations of 4% have been shown an increase in feed intake, growth rate and biomass production. The prawn *Artemesia longinaris* is a commercially important marine species that inhabits coastal waters of Argentina, Uruguay, and South Brazil, where temperatures range from 8 to 22ºC and salinities between 33 and 36 (Boschi & Gavio, 2005). Recently the interest in this shrimp has been increasing as a potential species for culture in temperate-zone. In the present study, we have analyzed water-soluble extracts of brown seaweeds *Undaria pinnatifida* and *M. pyrifera*, and their chemical characteristics and antioxidant properties *in vitro*. They were used as feed additives to *A. longinaris,* and the radical scavenging activity was investigated in midgut gland homogenates using electron paramagnetic spin resonance (EPR) spectroscopy.

MATERIALS AND METHODS

Experimental animals

Feeding trials were carried out with 150 juveniles held in 150 L glass aquaria with an under-gravel filter and a sand and crushed shell bed, at a density of 10 per m^2. Prawns were obtained from a commercial fisherman in the coastal waters of Mar del Plata, Argentina (38°S, 57º33'W). Experimental conditions were as follows: photoperiod, 12 h light/12 h dark; temperature, 20 ± 0.8°C; salinity 33; and pH 7.

Preparation of water-soluble extract of brown seaweeds

Fine powder of *U. pinnatifida* and *M. pyrifera*, were obtained from Soriano S.A (Gaiman, Chubut, Argentina). Hot-water extracts of algae were prepared based on the method of Fujiki *et al*. (1992). Ten grams of the milled fronds were added to 300 mL of deionized water and heated to boiling under reflux for three hours. The suspension was filtered through a sintered glass mesh (15 µm). The filtrate was concentrate under reduced pressure in a rotavapor. The hot water extract was dried and stored at -20ºC until use.

IR spectroscopy

FT-IR (spectral region spectra 4000-400 cm^{-1}, resolution 2 cm^{-1}) of the solid samples (water-soluble extracts) in the form of KBr tablets were recorded on a Perkin Elmer Spectrum BX spectrophotometer (FTIR) using Spectrum V 5.3.1 software.

Chemical analysis

Hydrolysis of polysaccharides was carried out with 2M CF_3COOH (90 min, 120ºC). The hydrolysis was performed by triplicate. Hydrolysates were derivatized to the alditol acetates with acetic anhydride-pyridine (Albersheim *et al*., 1967) and analyzed by CGL using a capillary column SP 2330 (Supelco) (30×0, 25 mm) on a HP- 5890 Gas Chromatograph equipped with a flame ionization detector (FID). Nitrogen was used as the carrier gas, with a head pressure of 15 psi and a split ratio of 100:1. Chromatography runs were isothermal at 230ºC, while the injector and detector were set at 240ºC. When a confirmation of identity was needed, the CGL/MS analyzed was carried out on Shimadzu QP 5050 A GC/MS apparatus working at 70 eV in similar conditions to those described above, but using He as carrier gas with a split ratio 60:1. Inositol was utilized as an internal standard.

Contents of fucoidans was estimated from the content of fucose+galactose, based on a 2 factor used to estimate the content of these polysaccharides (Larsen, 1978). Uronic acids were determined by the method of Filisetti-Cozzi & Carpita (1991). When the sample was insoluble, it was rendered soluble with sulfuric acid (Ahmed & Labavitch, 1977).

Feeding trials

Two experiments were performed: one of the trials consisted of three dry pelletized feeds (Table 1) supple-

Table 1. Ingredient composition of control feed. [a]Agustinier S.A. Mar del Plata, Argentina, [b]Melrico S.A. Argentina, [c]g kg^{-1}: cholecalciferol 1.8, thiamin 8.2, riboflavin 7.8, pyridoxine 10.7, calcium panthothenate 12.5, biotin 12.5, niacin 25.0, folic acid 1.3, B12HCl 1.0, ascorbic acid (Rovimix Stay C) 39.1, menadione 1.7, inositol 0.3, choline chloride 0.2, tocopherol acetate 75, vitamin A acetate 5.0.

Ingredient (g 100 g^{-1})	C	M1	M2	U1	U2
Fishmeal (65% crude protein)[a]	48.0	48.0	48.0	48.0	48.0
Soybean meal (42% crude protein)[b]	17.0	17.0	17.0	17.0	17.0
Corn starch	20.0	20.0	20.0	20.0	20.0
Squid protein (85% crude protein)	1.0	1.0	1.0	1.0	1.0
Wheat bran	8.5	7.5	6.5	7.5	6.5
Extract of *M. pyrifera*	0	1	2	0	0
Extract of *U. pinnatifida*	0	0	0	1	2
Fish oil	2.0	2.0	2.0	2.0	2.0
Fish soluble	2.0	2.0	2.0	2.0	2.0
Soybean lecithin	0.5	0.5	0.5	0.5	0.5
Cholesterol	0.5	0.5	0.5	0.5	0.5
Vitamins supplement[c]	0.5	0.5	0.5	0.5	0.5
Proximate composition (% dry matter)					
Moisture	5.5	5.5	5.5	5.5	5.5
Crude protein	41.0	41.0	41.0	41.0	41.0
Total lipid	11.8	11.8	11.8	11.8	11.8
Ash	9.4	9.4	9.4	9.4	9.4

mented with 0% (Control), 1% (M1), and 2% (M2) of extract of *M. pyrifera*, and in the other experiment, feeds with 0% (Control), 1 % (U1), and 2% (U2) of extract of *U. pinnatifida* were used. Formulations were stablished according to the chemical composition of the ingredients in order to obtain isoproteic and isolipidic diets. The chemical composition of the formulated feeds was determined through the proximate analysis (Table 1) according to AOAC (2003). All ingredients were mixed and cold pelletized (<50°C) by extrusion (Díaz & Fenucci, 2002). The pellets were oven-dried at 50°C for 24 h. Animals were fed *ad libitum* once a day. Feeding rate was adjusted daily in each tank in order to maintain feed waste at a minimum. Diets were tested in four replicate groups during 30 days. Individual prawns were weighed at the beginning and at the end of 30 days trials. At the end of the experiment, animals were anesthetized on ice and midgut glands were pooled for EPR analysis. Growth performance and survival were measured in terms of final individual weight, percentage of weight gain [[(final mean weight - initial mean weight)/initial mean weight] × 100], and percentage of survival.

DPPH radical assays

Radical scavenging activities were estimated for water-soluble extracts of two seaweeds and for midgut gland samples of experimental animals. DPPH radical scavenging activity was measured following the methodology of Díaz *et al*. (2014) using EPR with a Bruker Elexsys E 500 T spectrometer, operating at X-band. DPPH proved to be a very stable free radical (Díaz *et al*., 2004). A chloroform solution of each sample (50 µL) was added to 50 µL of DPPH (2.8×10^{-5} M) in chloroform under an argon atmosphere. After mixing, the solutions were transferred to quartz tubes with an internal diameter of 4 mm to achieve an adequate tuning at a constant room temperature of 293ºK and fitted into the cavity of de EPR spectrometer. The samples were analyzed at fixed time intervals during 30 min. Amplitude signals were transformed in radical concentrations by comparing the area under the EPR absorption spectrum of the sample to that of a chloroform solution of the concentration standard, DPPH. All data were recorded at the same acquisition parameters: microwave power, 12.93 mW; microwave frequency, 9.71 GHz; modulation fre-quency, 100 kHz; attenuation, 12 dB; modulation amplitude, 100 mT; magnetic field center, 346.5 mT; sweep width, 10 mT; and sweep time, 60 s for three scans. Measurements were repeated at least three times to minimize random errors. The standard plotting program Origin Pro was used to fit all sets of points whenever necessary.

Statistical analysis

The analysis of variance and least significant difference tests were conducted to identify differences among means. Data were reported as the mean ± standard

deviation. The results of a χ^2 test for goodness of fit were computed. In all cases, significance was set as $P < 0.05$ (Sokal & Rohlf, 1995).

RESULTS

IR spectroscopy

IR analysis allowed identifying the characteristic bands of the compounds of the water-soluble extracts (Fig. 1). The signal around 3400-3200 cm^{-1} corresponds to stretching of hydrogen bonded OH groups. Bands at 1740 cm^{-1} were assigned to C=O of O-acetyl groups. Signals at 1638 cm^{-1} and 1420 cm^{-1} derived from the asymmetric and symmetric stretching vibration of carboxylate anion respectively. A band around 1457 cm^{-1} was assigned to the scissoring vibration of CH_2 (galactose, xylose) and asymmetric bending vibration of CH_3 (fucose, O-acetyls). The IR band in the 1380-1376 cm^{-1}spectral region was assigned to symmetric bending vibration of the methyl group and both algae exhibited a broad band around 1260-1220 cm^{-1}, atributed to the presence of sulfate ester groups (S=O) which is a characteristic component in fucoidans of brown seaweeds. Bands in the region 900-840 can be assigned to the presence of sulfated and non-sulfated galactose. The IR features at 625 and 580 cm^{-1} were attributed to the asymmetric and symmetric O=S=O deformation of sulfates. Bands located at 960 cm^{-1} was related to C-O stretching vibrations with contributions from C-C stretching and C-C-O deformation vibrations of the uronic acids residues in heteropolymeric blocks.

Chemical analysis and DPPH radical assays of extracts

The monosacharide composition of the *U. pinnatifida* and *M. pyrifera* water-soluble extracts was determined by CGL-MS (Table 2).

The results showed that the main chemical components of all samples were mannitol and fucoidans. Both samples present similar composition, the main alditol determined in the hydrolyzate was mannitol, although it can be either originated in the reduction of mannose or exist as mannitol originally present in the samples, the second chance appears to be more possible.

Other important monosaccharides were fucose and galactose in *U. pinnatifida* extract, and fucose in *M. pyrifera* extract. Also in both cases minor amounts of monosacharides such as rhamnose, glucose, xylose and arabinose were determined. The content of fucose+galactose was higher in *U. pinnatifida*; the percentages were 5.9 and 8.7% for *M. pyrifera* and *U. pinnatifida*, respectively. These values correspond to 11.8 and 17.4% of fucoidans. The fucose/galactose ratio was 3.5 for *M. pyrifera* extract and 1 for *U. pinnatifida*. In Fig. 2 the kinetic of the DPPH reaction of polysaccharide extracts of *M. pyrifera* and *U. pinnatifida* can be observed, scavenging activity was detected in both extracts, the signal decayed to 50 and 62% in 18 min for *U. pinnatifida* and *M. pyrifera*, respectively.

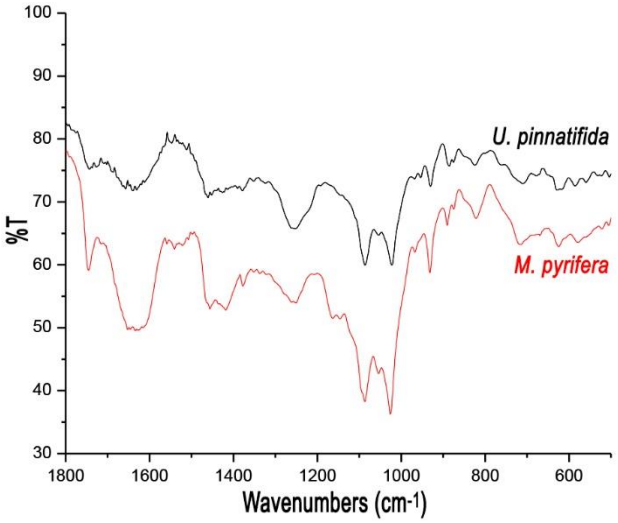

Figure 1. Infrared analysis of water-soluble extracts of brown seaweeds showed between 1800 and 500 cm^{-1}.

Feeding trials

Weight gain and survival data for prawns fed with different feeds are presented in Table 3, after 30 days of experimentation, percentage of increment in mean weight of prawns under different treatments varied between 8.5 and 16.7%, and there were significant differences between animals fed with feeds added with *U. pinnatifida* extract (U1 and U2) and the other treatments.

Survival ranged between 60 and 79%, with no significant differences among them. Reaction kinetics of the DPPH with midgut gland homogenates of prawn *A. longinaris* fed with 0%, 1% and 2% of extracts of *M. pyrifera* and *U. pinnatifida* are shown in Fig. 3a and 3b, respectively. All treatments showed antioxidant protective capacity as evidenced by the ability to react with the radical DPPH. In prawns fed with M2, it was observed a rapid radical decay in the first 2 min of reaction, detected 60% of remnant DPPH, while for those fed with the control and M1 feeds remnants of 88 and 81.9% were recorded, respectively (Fig. 3a). Supple-mentation with 1 and 2% of *U. pinnatifida* showed similar quenching capacity of homogenates (Fig. 3b) than those fed with M2 at two minutes of reaction. On the other hand, in animals fed with feeds added with *U. pinnatifida* extract DPPH signal decayed and was effectively consumed in about 30 min (2%,

Table 2. Water-soluble extracts monosaccharide composition (g 100 g^{-1} sample) of brown seaweeds after hydrolysis. [a]By the current method, mannose cannot be distinguished from free mannitol. *tr*: traces, <0.1%.

	Rhamnose (%)	Fucose (%)	Arabinose (%)	Xylose (%)	Mannose[a] (%)	Galactose (%)	Glucose (%)
M. pyrifera	0.2	4.6	0.4	1.3	16.8	1.3	0.3
U. pinnatifida	*tr*	4.4	0.2	0.2	14.5	4.3	0.5

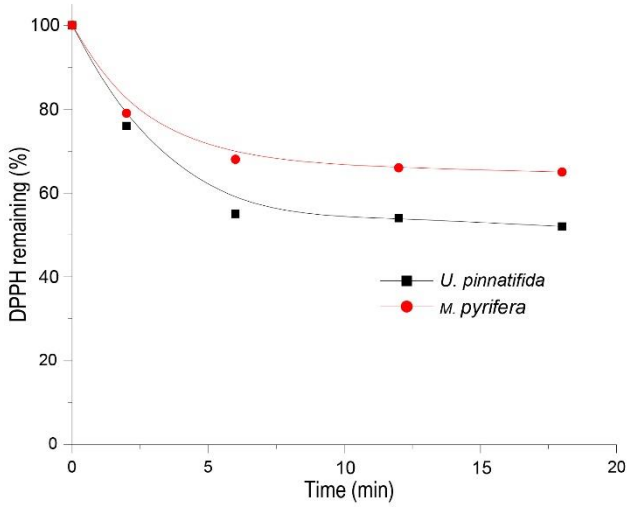

Figure 2. Brown seaweeds water-soluble extract, DPPH radical scavenging activities. Values are the mean of three determinations.

remained) while for control, M1 and M2 was 24% remained without reaction.

DISCUSSION

Compounds from marine algae have been used as gelling, thickening, and emulsifying agents in a range of food products, but were not identified as a source of health-promoting compounds. Recent functional food ingredient research has shown that marine algae are a rich source of nutraceuticals with a variety of biological activities (Vo & Kim, 2013). In differents studies has been reported that polysaccharides present in algae or their extracts (alginate, fucoidan, and laminarin) can control viral and bacterial diseases (Holdt & Kraan, 2011). The polysaccharides extraction with acid or water as solvent can be not selective, obtaining complex mixtures of the major compounds present in brown macroalgae (Kadam *et al*., 2013). In the present study, we prepared water-soluble extracts from seaweeds to use the polysaccharide bioactive fraction as functional food. The CGL-MS analysis determined that the main chemical components of all samples were mannitol and fucoidans. The uronic acid present can be part of the fucoidan molecules (Bilan *et al*., 2010) or become from co-extracted alginate.

The IR analysis showed differents absortion bands that support the presence of monosacharides and polysacharides. Band at 960 cm^{-1} was related to stretching vibrations with contributions from deformation vibrations of the uronic acids residues in heteropolymeric blocks (Leal *et al*., 2008). The signal at 625 and 580 cm^{-1} was attributed to the asymmetric and symmetric O=S=O deformation of sulfates (Sekkal & Legrand, 1993) and the presence of sulfated was confirmed by infrared spectra in both water-soluble extracts due to the characteristic absorption band at 1230 cm^{-1} (Nikonenko *et al*., 2000; Synytsya *et al*., 2010). These polysaccharides usually contain large proportions of L-fucose and sulfate, together with minor amounts of other sugars like xylose, galactose, mannose and glucuronic acid (Ponce *et al*., 2003). Large variations are observed between species, which have an impact on determination of the polysaccharide structure. Our results demonstrated that the components of water-soluble extracts isolated from *M. pyrifera* had different composition to those of *U. pinnatifida*, with higher fucoidan content in the latter. Fucoidans present in brown algae can be considered as potent natural antioxidants (Rupérez *et al*., 2002; Rocha De Souza *et al*., 2007; Wang *et al*., 2010) and their antioxidant activity depends on their structural features such as degree of sulfation, molecular weight, type of the major sugar and branching. For example, low molecular weight sulfated polysaccharides have shown better antioxidant activity than those of high molecular weight, this fact can be explained because smaller polysaccharides may incorporate into the cells more efficiently and donate protons more effectively compared to larger polysaccharides (Wijesecara *et al*., 2011). Our data showed that the water-soluble extracts isolated from *M. pyrifera* and *U. pinnatifida* have different relation fucose/galactose. This relation corresponds with fucoidan content which was higher in *U. pinnatifida* and the values were similar to those reported in previous studies (Synytsya *et al*., 2010; Hemmingson *et al*., 2006). The kinetic of the DPPH

Table 3. Average weight initial, weight gain, and survival rate of *A. longinaris* fed with feeds containing polysaccharide extracts at various concentrations for 30 days. IMG: initial mean weight, FMW: final mean weight; GWP: gain weight, percentage, S: survival. Means in a row with different superscript letters significantly differ ($P < 0.05$).

	C (control feed)	Feeds with extract of *M. pyrifera*		C (control feed)	Feeds with extract of *U. pinnatifida*	
		M1	M2		U1	U2
IMG (g)	5.6 ± 0.93	5.9 ± 0.73	5.8 ± 0.70	3.78 ± 0.67	3.76 ± 0.73	3.46 ± 0.48
FMG (g)	6.1 ± 0.8	6.44 ± 0.72	6.27 ± 0.92	4.13 ± 0.17	4.31 ± 0.26	4.04 ± 0.74
GWP (%)	8.5[a]	9.2[a]	8.66[a]	9.26[a]	14.63[b]	16.76[b]
S (%)	67.8[c]	66.7[c]	79.2[c]	60[c]	60[c]	73.3[c]

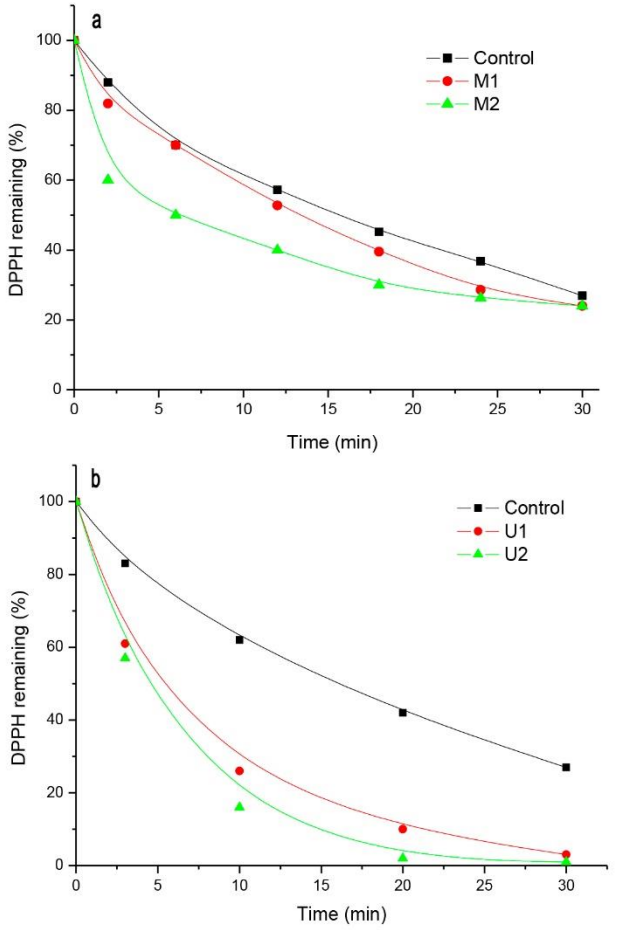

Figure 3. Reaction kinetics of tissue with DPPH radical. Tissue extracts come from a pool of animals fed pelletized feeds added with water-soluble extracts. All tests were conducted in triplicate and the means are used. a) Animals fed with *Macrocystis pyrifera* extract, b) animals fed with *Undaria pinnatifida* extract.

reaction of polysacharides extract showed that the highest activity was determined in *U. pinnatifida*.

In aquaculture, one of the great challenges is to optimize the supplied food (Akiyama *et al*., 1991; Suresh, 2006). The demand for balanced feed for shrimp culture has a growing trend in the world, so finding new non-conventional ingredients like seaweeds, becomes important. Several works (Rivera *et al*., 2002; Gutiérrez-Leyva, 2006; Cruz-Suárez *et al*., 2009) evaluated the effect of including *M. pyrifera* meals in feeds for white shrimp *Litopenaeus vannamei*, with excellent results in terms of growth rate and survival. In the present study supplementation with polysaccharides extracts of seaweeds, *M. pyrifera* and *U. pinnatifida* showed in all treatments an acceptable survival for commercial crops (higher than 60%) (Clifford, 1994), with similar values than those obtained for other species of shrimp (Casas-Valdez *et al*., 2006; Cruz-Suárez *et al*., 2009). Studies reported that inclusion of brown algae in addition to improving growth, feed intake and feed texture, reduce dry matter loss and increase water absorption, proving to be an excellent binder for pelletized feeds (Cruz-Suárez *et al*., 2007, 2009). Moreover, Niu *et al*. (2015) reported that inclusion of *U. pinnatifida* meal in the diets of *Penaeus monodon*, promotes increase of final average weight values, weight gain and specific growth rate. In the present work, prawns fed feeds U1 and U2 showed a higher growth; added *M. pyrifera*, although it was no better growth, nor rejection effect thereof which occurs when the flavor and palatability of food is affected (Syed & Suresh, 2002).

The antioxidant activity of both extract was tested for midgut gland homogenates by electron resonance spectroscopy, in prawns fed with 0%, 1% and 2% of extracts of *M. pyrifera* and *U. pinnatifida*. All treatments showed antioxidant protective capacity as evidenced by the ability to react with the radical DPPH. The difference in the antioxidant activity of the extract of *U. pinnatifida* regard to *M. pyrifera* could be adjudged to the greater amount of sulfated polysaccharides, which act as natural antioxidants. Potential of marine algae as a functional food ingredient is become important in aquatic animals, results obtained so far, indicated that could be a

promising ingredient for better health status of animals under culture. Previous studies have evaluated supplementation of diets for shrimp with several levels of algal meals, 2-8% of *M. pyrifera* (Cruz-Suárez *et al*., 2009), 2-4% of *Sargassum* spp. (Cruz-Suárez *et al*., 2007) and 1-6% of *U. pinnatifida* (Niu *et al*., 2015) obtaining excellent results in terms of growth. On the other hand, extracts of different algae are used to improve the antioxidant capacity; so to better exploit this potential, is necessary the of use unconventional extraction technologies, which do not utilize organic solvents, though the extracts obtained are not pure. The extraction technique used by us is simple, non-toxic, ecofriendly, and showed good experimental results regarding the analysis of antioxidant capacity, due to high concentration of fucoidan in seaweed extracts. Results of this investigation show that the use of polysaccharide extracts of *M. pyrifera* and *U. pinnatifida* is feasible as additive in diets for the prawn *A. longinaris*. The optimal level of inclusion may vary depending on the algal species, for the cultivation of this species, the recommended concentration should be 2%, since it is the one that produces the best antioxidant capacity, although the growth with performance was improved also significantly only *U. pinnatifida* extract.

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